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Mutational analysis of primary alcohol metabolism in the methylotrophic actinomycete *Amycolatopsis methanolica*

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Abstract

Mutants of the methylotrophic actinomycete *Amycolatopsis methanolica* unable to grow on methanol as carbon source were isolated and characterized. Mutants specifically affected in methanol utilization were deficient in formaldehyde assimilation. Mutants blocked in the first step of primary alcohol oxidation (C1–C4) had lost activity of the tetrazolium-dependent alcohol dehydrogenase, a three-component enzyme complex. This complex, or individual components, thus play a crucial role in utilization of primary alcohols in *A. methanolica*.

Keywords: Actinomycete; *Amycolatopsis methanolica*; Primary alcohol; Ethanol; Methanol; Mutant; Mutagenesis

1. Introduction

Methanol oxidation in Gram-positive bacteria involves cytoplasmic NAD(P)-dependent alcohol dehydrogenase enzymes. *Bacillus methanolicus* employs a methanol dehydrogenase (MDH) with 10 subunits of 43 kDa, each containing a tightly (but noncovalently) bound NAD(H) cofactor [1]. Studies with purified MDH showed that the cofactor NAD is reduced upon methanol addition [2]. MDH displayed a very low methanol oxidizing activity when incubated with methanol and coenzyme NAD; very high activities were observed following addition of a 29 kDa *B. methanolicus* protein [3]. In vivo this activator protein may re-oxidize the MDH-bound NADH cofactor [2].

Structurally related decameric proteins (49 kDa subunits), showing clear N-terminal amino acid similarity with MDH of *B. methanolicus*, subsequently have been purified to homogeneity from the actinomycetes *Amycolatopsis methanolica* and *Mycobacterium gastri* [4,5]. These proteins oxidize several primary alcohols, using the artificial electron acceptor *N*,*N*-dimethyl-*p*-nitrosoaniline (NDMA), displaying for instance methanol:NDMA oxidoreductase (MNO) activity [4,5]. The MNO enzymes possess tightly (but noncovalently) bound NADP(H) cofactors but completely lack methanol oxidizing activity with coenzyme NAD(P). We failed to identify an associated activator protein in *A. methanolica*; also the *B. methanolicus* activator protein failed to stimu-

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late activity of MNO of *A. methanolica* (unpublished data) [4,5].

Also 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) [6] and 2,6-dichlorophenolindophenol (DCPIP) [7] function as electron acceptors for methanol oxidation in *A. methanolica* extracts. Attempts to purify these DCPIP-ADH or MTT-ADH activities revealed protein complexes in both cases [7,8].

Also an ethanol:NDMA oxidoreductase (ENO) protein, active with several primary alcohols but not with methanol, has been purified from *A. methanolica* [9]. The ENO enzyme, thought to function during growth on primary alcohols other than methanol, has three subunits of 39 kDa each with a tightly bound NAD cofactor. No N-terminal amino acid sequence similarity was observed with MNO [9].

The in vivo roles of MNO and the MTT-ADH complex in alcohol oxidation in *A. methanolica* were studied by evaluating the induction patterns of these enzymes, and by a mutational analysis of methanol metabolism.

2. Materials and methods

2.1. Microorganism and cultivation

Strain WV2, a plasmid pMEA300-free derivative of *A. methanolica* wild-type (NCIB 11946) [10], was cultivated as described [11,12]. Carbon-limited chemostats were run at a constant temperature of 37°C and pH 7.0, automatically adjusted with 1 M NaOH. Gelrite was used in solid media with methanol (500 mM), ethanol (100 mM), propanol (50 mM) or butanol (50 mM) as carbon sources. Acetate (30 mM), betaine, xylose and mannose (all at 50 mM) were also used as carbon sources.

2.2. Mutant isolation procedures

Glucose-grown cells of *A. methanolica* were sonicated to obtain single cells [13]. A thin layer of cell suspension was subjected to UV irradiation (Philips TAW 15 W) at a distance of 20 cm for 25 s, yielding a killing of 80%. Dilutions were spread on methanol Gelrite plates and pinpoint colonies selected. Stable mutants which grew very poorly on methanol Gelrite plates and showed good growth on glucose and ethanol were used for further studies. This procedure exclusively yielded mutants blocked in one-carbon assimilation. Following UV irradiation, cells were also spread on glucose agar plates; after colonies had formed, replica plating was performed onto methanol Gelrite plates. Stable mutants which showed no (or very poor) growth on methanol Gelrite were used for further studies. This procedure yielded mostly mutants blocked in primary alcohol utilization. With both methods, about 0.1% of the cells surviving the mutagenic treatment were affected in methanol utilization.

2.3. Induction of methanol metabolism

Methanol-negative mutants still able to grow on ethanol were induced in batch culture during growth on a mixture of ethanol (50 mM) and methanol (50 mM). Methanol/ethanol-negative mutants were induced by adding methanol (30 mM) to the medium reservoir bottle of glucose-limited (5 mM) steady state chemostat cultures ($D = 0.10\ h^{-1}$).

Utilization of formaldehyde (a very toxic compound) was tested in a glucose-limited chemostat cultures by gradually replacing glucose in the feed by formaldehyde [14]. The formaldehyde concentration in the feed was increased with 5 mM every two dilutions. When reaching a concentration of 5 mM glucose and 25 mM formaldehyde, a new medium reservoir was installed with 50 mM formaldehyde as sole carbon source ($D = 0.05\ h^{-1}$).

Growth on mixtures of 10 mM glucose and 50 mM methanol was studied in a batch fermenter with automatic pH adjustment and aeration at 2 l/min. Every 2 h samples were analysed for OD430, substrate concentrations and enzyme activities.

2.4. Enzyme assays

Cells grown in batch cultures were harvested in the mid-exponential growth phase; cells grown in chemostat cultures were harvested at steady state. Cells were disrupted by French pressure cell treatment [12]. The crude extract was centrifuged for 30 min at 4°C at $40\,000 \times g$, resulting in cell-free extract. The activities of MNO [4], MTT-ADH [6], DCPIP-ADH

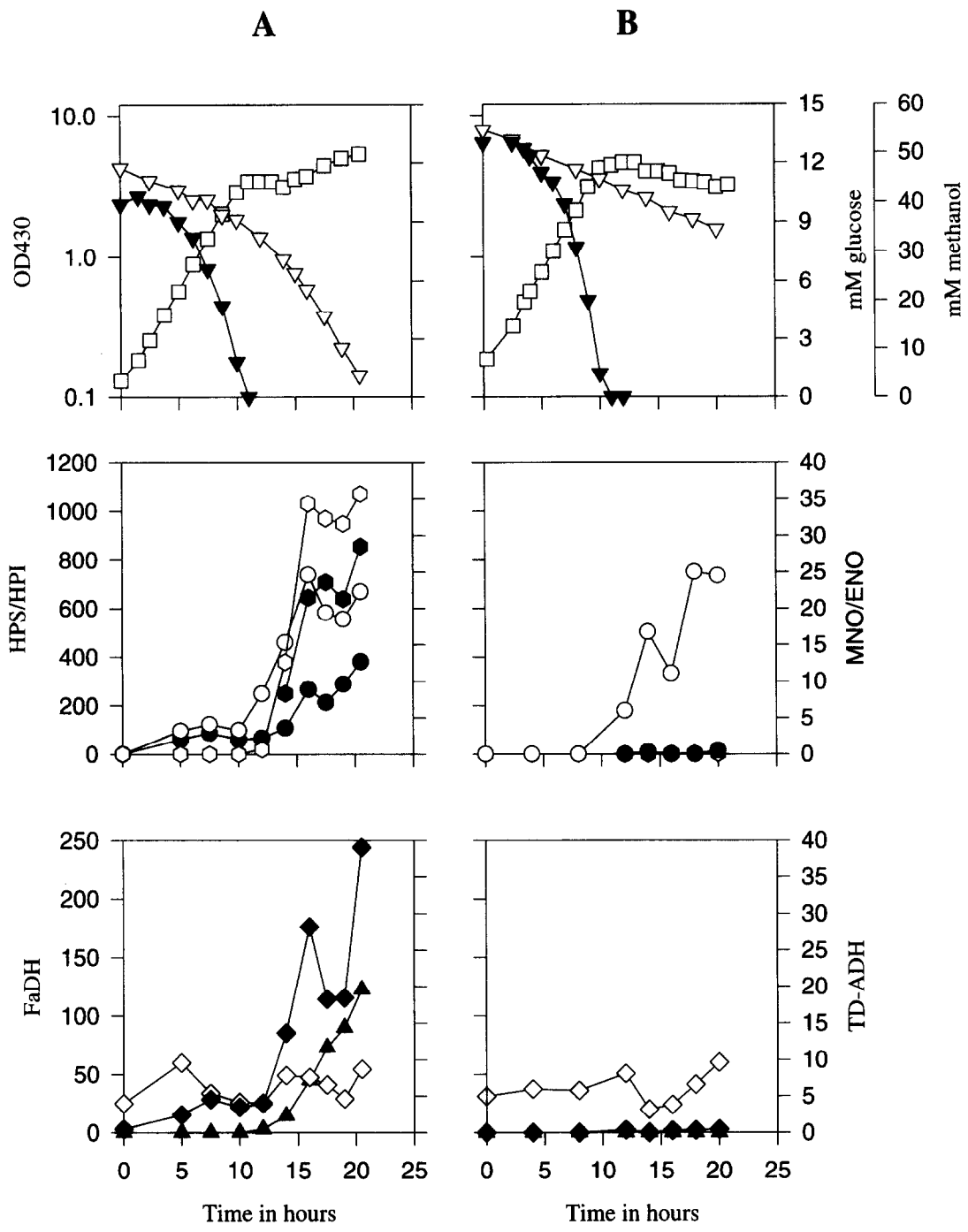

Fig. 1. Growth of wild-type *Amycolatopsis methanolica* strain WV2 (A) and mutant strain MM27 (B) on mixtures of glucose and methanol in batch culture. Specific activities are in mU/mg protein. (□) OD430, (▼) glucose, (▽) methanol, (●) MNO, (○) ENO, (empty hexagons) HPS, (filled hexagons) HPI, (◆) NAD-FaDH, (◇) DL-AlDH, (▲) MTT-ADH.

Table 1
Phenotypes of the different classes of mutants of *Amycolatopsis methanolica* strain WV2 blocked in methanol utilization

Mutant	Growth on			Enzyme activities				Number of mutants
	MeOH	EtOH	Fald[a]	HPS	HPI	MNO	MTT-ADH	
Wild-type	++	++	++	++	++	++	++	
Class 1	±	++		-		++	++	A: 22
								B: 7
Class 2	±	++		++		++	++	A: 2
								B: 1
Class 3	-	-	++	++	++	-	-	B: 7
Class 4	-		++	++	++	++		B: 1

A, selected as methanol-negative, ethanol-positive mutants; B, selected as methanol-negative mutants. MeOH, methanol; EtOH, ethanol; Fald, formaldehyde; HPS, hexulose-6-phosphate synthase; HPI, hexulose-6-phosphate isomerase; MNO, methanol:NDMA oxidoreductase; MTT-ADH, MTT-dependent alcohol dehydrogenase.
[a]In formaldehyde-limited chemostat cultures.

[7], ENO [9], NAD-formaldehyde dehydrogenase (NAD-FaDH) and dye-linked aldehyde dehydrogenase (DL-AlDH) [15], and hexulose-6-phosphate synthase (HPS) and hexulose-6-phosphate isomerase (HPI) [16], were measured at 30°C. Partial purification of the protein components of the MTT-ADH complex and reconstitution studies were performed as described [8]. Respiration studies with whole cells, using final substrate concentrations of 100 mM methanol, 50 mM ethanol or 5 mM formaldehyde, were as described [11].

3. Results and discussion

3.1. Induction patterns of alcohol-oxidizing enzymes

The activity levels of the various alcohol oxidizing enzymes reported to be present in *A. methanolica* were studied in cells grown on various carbon sources. MNO activity was found only in cells grown on primary alcohols (C1–C4), formaldehyde, acetate or betaine. Betaine degradation results in generation of formaldehyde [17]. In general, growth under car-

Table 2
Characteristics of mutants of *Amycolatopsis methanolica* strain WV2 blocked in methanol utilization (A, cells harvested from a steady-state, formaldehyde-limited (S_r 50 mM) chemostat culture, at a dilution rate of 0.05 h^{-1}; B, cells harvested from a steady-state, glucose-limited (S_r 5 mM) chemostat culture, at a dilution rate of 0.10 h^{-1}, induced for 4 dilutions with methanol (S_r 50 mM)

A		WV2	MM27	MM31	MM36	MM49
Activities (mU/mg)						
MNO		7.5	< 0.5	< 0.5	< 0.5	5.1
TD-ADH		8.1	< 0.5	< 0.5	< 0.5	< 0.5
Oxidation rates (nmol O_2/min per mg)						
Methanol	V_{max}	60	0	6	7	0
Ethanol	V_{max}	130	0	12	10	0
Formaldehyde	V_{max}	140	215	180	550	120
	K_S (μM)	145	400	300	1900	

B	WV2	MM27	MM31	MM32	MM36
OD430	3.4	1.8	1.4	1.4	0.8
MNO	8.4	< 0.5	< 0.5	< 0.5	1.5
ENO	28.7	15.8	16.3	21.8	31.0
MTT-ADH	18	< 0.5	< 0.5	< 0.5	< 0.5
DCPIP-ADH	2	< 0.5	< 0.5	< 0.5	< 0.5

bon-limitation in chemostat cultures resulted in derepression of MNO, even with glucose as carbon source. The presence of MTT-ADH activity was more restricted and only detected in cells grown on primary alcohols or on formaldehyde. MTT-ADH (and MNO) activity could be further induced by adding methanol to the feed of a glucose-limited chemostat cultures. DCPIP-ADH activity was only detected in cells grown on methanol. ENO activity was detected following growth on primary alcohols, betaine, xylose and mannose. Growth under carbon-limitation resulted in derepression of ENO.

3.2. Glucose repression of methanol utilization

Diauxic growth was observed in batch culture when inoculating glucose-pregrown cells in mineral medium with methanol plus glucose (Fig. 1A). Initially, only glucose was consumed, while the methanol concentration decreased slowly, due to evaporation. Glucose depletion was followed by a 3 h lag phase and by growth on methanol. During growth with glucose, the MNO and MTT-ADH activities remained very low; following glucose depletion, both activities increased strongly during growth on methanol. Both MNO and MTT-ADH thus appear relevant for methanol metabolism. NAD-FaDH became clearly induced during growth with methanol; the DL-AlDH activity profile suggests that this is a constitutive enzyme. As expected [14], the key enzymes of the RuMP cycle of formaldehyde assimilation, HPS and HPI were absent during growth with glucose and strongly increased during methanol utilization (Fig. 1A).

3.3. Growth on solid media

Effective methods for the isolation of amino acid auxotrophic mutants of *A. methanolica* have been developed [13]. The isolation of mutants unable to grow on methanol as sole carbon source caused some problems, however. *A. methanolica* was unable to form colonies of a reasonable size, with a diameter of more than 1 mm, on methanol agar plates. The very tiny colonies which appeared upon prolonged incubation were comparable in size to colonies on agar plates minus carbon source. This poor growth probably occurs on (impurities in) the agar, apparently also effectively repressing methanol metabolism. Several agar alternatives were tested, such as washed agar, very pure noble agar and about 10 different types of agarose; none of these gave a positive result, however. Only the use of Gelrite as agar substitute and mineral medium with 500 mM methanol yielded larger size colonies that could be clearly distinguished from the tiny colonies formed on Gelrite medium minus carbon sources.

3.4. Mutagenesis and mutant isolation

A total of 24 mutants forming pinpoint colonies on methanol Gelrite but able to grow normally on ethanol were further studied. Only mutants that had lost HPS (class 1, 22 mutants) or HPI (class 2, 2 mutants) were identified (Table 1). These mutants thus are unable to grow on methanol because of a block in the RuMP cycle, the single-carbon assimilatory pathway used by *A. methanolica* [14]. A possible explanation for the failure to isolate mutants blocked in methanol oxidation via this procedure is that the same enzymes are involved in methanol and ethanol catabolism.

The replica plating technique yielded 16 mutants growing normally on glucose agar plates but unable to form colonies on methanol Gelrite. Eight of these mutants grew normally on ethanol and were identified as class 1 mutants (7; HPS negative) and class 2 mutant (1; HPI negative) (Table 1). The eight other mutants were also completely impaired in growth on ethanol; mutants that had lost MTT-ADH plus MNO (class 3, 7 mutants) or MTT-ADH only (class 4, 1 mutant) were identified (Table 1).

3.5. Mutant characterization

The class 3 mutants MM27, MM31, MM32, MM36, and the class 4 mutant MM49 (Table 1), were studied in more detail. All 5 mutants had lost ability to grow on methanol, ethanol, propanol and butanol; they were still able to grow on formaldehyde, indicating that the mutations only affected the first step, conversion of methanol to formaldehyde (Table 2A). All 5 mutants possess ENO activities comparable to strain WV2; nevertheless, they are unable to grow on ethanol (Table 2B). MNO rather than ENO thus has a key role in ethanol metabolism

(and in that of other primary alcohols). None of the mutants possessed DCPIP-ADH activity (Table 2B). All 5 mutants appeared quite stable, even allowing growth under selective stress in chemostat cultures without reverting (Table 2). Strain MM27 reverted at a frequency of 10^{-10} on methanol Gelrite plates. With difficulty, several revertants of strain MM27 able to grow on methanol Gelrite or on ethanol Gelrite were isolated. Five revertants of each class were characterized, revealing that all 10 had regained MNO as well as MTT-ADH activity simultaneously. It thus appears that the loss of both MTT-ADH and MNO activities in strain MM 27 is due to a single mutation, affecting growth on methanol as well as on other primary alcohols.

Mutant MM27 was also incubated on a mixture of glucose and methanol in batch culture. Strain MM27 grew normally on glucose, but after glucose depletion no growth on methanol occurred and no methanol consumption was observed (Fig. 1B). MNO, MTT-ADH, NAD-FaDH, and the formaldehyde assimilating enzymes (HPS, HPI), remained undetectable. Interestingly, ENO activity did appear in this second phase. The absence of HPS and HPI in the second phase might be due to lack of induction, since we subsequently observed that MM27 cells grown on formaldehyde did possess HPS/HPI.

Four different formaldehyde oxidizing enzymes have been purified from *A. methanolica*. The in vivo roles of these enzymes remain to be established. DL-AlDH [18] appears to be constitutive whereas NAD-FaDH is inducible in strain WV2 (Fig. 1A). The NAD-FaDH assay used in the present study was performed with crude extracts and thus detects both NAD-dependent aldehyde dehydrogenase [15] and factor-dependent formaldehyde dehydrogenase [19] activities. The absence of NAD-FaDH activity in strain MM27 (Fig. 1B) is quite surprising; neither of the two enzymes contributing to this activity thus appears essential for formaldehyde oxidation. Either DL-AlDH [18] or formate ester dehydrogenase [20] thus may play a major role in formaldehyde oxidation.

Compared to strain WV2, whole cells of the mutants displayed a minimal capacity to oxidize methanol and ethanol (Table 2A). Formaldehyde oxidation generally remained high. Interestingly, strain MM36 showed a very high capacity to oxidize formaldehyde but the affinity for this substrate was severely reduced.

The Class 4 mutant MM49 still possessed MNO activity but had lost MTT-ADH activity. Recently, Bystrykh et al. [8] provided biochemical evidence that MTT-ADH is a three-component protein complex, with the MNO protein, a high (H protein) and a low (L) molecular mass protein as components essential for MTT-ADH activity. Accordingly, mutants blocked in MNO also have lost MTT-ADH activity (this study). We now observed that in vitro MTT-ADH activity of mutant MM49 could be restored by adding partially purified component H from strain WV2, indicating that MM49 lacks a functional H-component.

In conclusion, our mutant data provide clear evidence that the MNO and H protein components of the protein complex with MTT-ADH activity play a crucial role in utilization of primary alcohols (C1–C4) in *A. methanolica*. MNO may play a role in the initial oxidation of these alcohols. The role of the H protein remains to be established.

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